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New Materials and Methods for Hierarchically Structured Tissue Scaffolds

Benita M. Comeau[1], Yusif Umar[2], Kenneth E. Gonsalves[2], and Clifford L. Henderson[1*]
[1]School of Chemical & Biomolecular Engineering, Georgia Institute of Technology
Atlanta, GA 30332-0100, U.S.A.
[2]Department of Chemistry, University of North Carolina-Charlotte
Charlotte, NC 28223, U.S.A.
* Corresponding Author – Email: cliff.henderson@chbe.gatech.edu

ABSTRACT

The overall goal of our work is to develop new methods and materials for the fabrication of hierarchically structured, three-dimensional (3D) tissue scaffolds. Conventional scaffolds commonly lack substantial mechanical strength, and there is difficulty in controlling porosity, pore distribution, and pore interconnectivity. Additionally, the chemical nature of these scaffolds is typically homogenous. The ability to chemically modify selected areas on a scaffold is one method to direct cell growth in deliberate patterns; which could aid in the engineering of complex, functioning tissues. The general aim of this work is to address these issues through the application of stereolithography (SL) to the fabrication of hierarchically structured scaffolds.

In order to achieve this goal, photopolymerizable materials must be developed that are both compatible with cell growth and with SL processing. SL methods are designed to produce arbitrary control over the physical structure of the part. In addition to physical structure control, control over the local surface chemistry of the scaffold is also desired. This would permit the use of both physical and chemical cues to control cell behavior in a tissue engineering construct. Chemical control could be achieved in SL methods by using photopolymerizable materials that can also be selectively chemically modified during the SL part building process. This paper provides an update on our work directed at using combined photoradical initiated polymerization and photoacid generator based chemical modification of a polymeric scaffold via multi-wavelength SL to produce hierarchically structured scaffolds.

INTRODUCTION

One prevalent tissue engineering approach is to (1) create a 3D scaffold, (2) seed it with the appropriate cells, growth factors, and other signaling cues, (3) allow the cells to grow and differentiate, and (4) finally implant this engineered tissue into a patient. A critical component to the success of this approach is the design and fabrication of the 3D scaffold. The scaffold must support many functions such as providing mechanical support and structure, facilitating nutrient and waste transport, and supplying physical and chemical cues to the growing cells. Many routes for creating a 3D scaffold have been studied, including 2D layer by layer methods [1], [2], direct fabrication of 3D forms through injectable hydrogels [3], polymer-porogen methods such as salt leaching [4], and rapid prototyping methods [5], [6]. For the first three methods, it is difficult to envision how one can create a complex hierarchical structure, either chemically or structurally. However, in this work it is proposed that by adapting stereolithography (SL), a rapid prototyping method, it should be possible to develop a method that allows for the direct fabrication of physically complex and chemically structured scaffolds using a single process.

SL is a fabrication method which uses a laser to "write" patterns into a vat containing a photopolymerizable resin. The first step in performing SL is generating a computer aided design file of the structure of the desired 3D object and slicing it into a series of 2D layers. The next step is to sequentially build up the desired part using SL layer-by-layer from the 2D slice data. In a typical SL tool, a movable stage (or "elevator") is located within the resin vat to support the part as it is being formed (Figure 1). To build the part, the stage is first brought within close proximity of the vat surface such that a thin layer (typically ~100 μm thick) of the resin covers the stage. The SL laser is then raster scanned over the layer in a patternwise fashion according to the 2D data for the bottom slice of the object, resulting in polymerization and solidification of the desired regions of that layer. Next, the stage is lowered into the monomer vat by the thickness of one layer, and the process is repeated with the next 2D data slice. Once the part is finished, it is removed from the vat and excess monomer is washed from the object. Following the washing step, post-processing may be done to improve the properties of finished object.

The goal of this work is to develop mixtures of biocompatible monomers and photoinitiators that can be used in conjunction with SL tools to create 3D scaffolds for tissue engineering applications. Furthermore, beyond the use of SL to control the physical structure of a polymer object or scaffold, this work is directed at developing strategies for using SL methods to also control the local chemical composition of the polymer scaffold surface. To that end, we investigated the use of biocompatible monomers containing hydroxyl groups that are reactively capped with a "protecting" group (e.g. t-butoxycarbonyl group) which can be selectively deprotected in certain areas using exposure to light in the SL tool. This deprotection chemistry is analogous to the strategy employed for making the "chemically amplified photoresists" (CARs) that are used in microlithography processes for semiconductor fabrication [7]. The deprotection reactions are generally catalyzed by acid, and thus the addition of a photoacid generator (PAG) to a protected monomer material permits local control of the polymer surface chemistry.

It has been previously shown that it is possible to produce the biological equivalent of a photoresist, referred to as a "bioresist", that can be used to direct cell growth on 2-D surfaces [8]. The material used in this previous work is composed of a copolymer of methyl methacrylate and 3-(t-butoxycarbonyl)-N-vinyl-pyrrolidone. A PAG can be incorporated with this polymer, and the mixture may be exposed and developed to generate topographic features, similar to a conventional CAR (Figure 2). By exposing the features and heating the sample, without development in aqueous base, the deprotection reaction occurs and modifies the polymer hydrophilicity, but no topographic pattern is produced. This hydrophilic pattern has been shown capable of directing patterned cell growth [8]. Using these materials to directly fabricate 3-D scaffolds however is challenging and requires methods analogous to the 2-D layer-by-layer assembly mentioned earlier. The use of similar materials for stereolithography could however present a more facile route to producing complex 3-D hierarchically structured scaffolds.

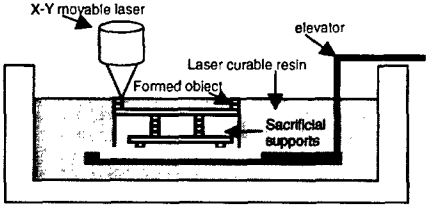

Figure 1. Stereolithography apparatus.

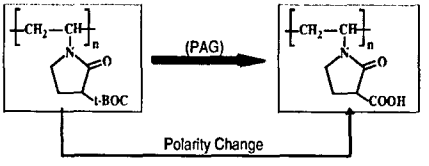

Figure 2. Basic "bioresist" chemistry.

There are two basic ways to adapt this idea for use in stereolithography. Both require that the material in the SLA vat include a blocked monomer, a photoinitiator, and a PAG (which must be active in a region of spectrum distinct from the photoinitiator). For the first method, a part may be made in the SL tool, and then subsequently exposed to UV light of another wavelength. This will generate a 3D structured part, with a chemically patterned surface (Figure 3). In addition to directing cell growth on the surface, this surface pattern may also be able to direct cell growth some depth into the scaffold.

The second method for using this materials strategy via SL would be to perform the two exposures in sequence while the part is being built. That is, each layer of the 3D part could be exposed to two light sources of different wavelengths. The first wavelength would polymerize the layer, while the second would initiate local deprotection. In this manner, each layer could be independently patterned in both shape and chemistry, creating a hierarchically structured part.

There are two main technical considerations that must be addressed to employ the bioresist concept in the SL system. First, the final part must be non-toxic. In the photopolymerization process, it is possible to trap small toxic molecules, such as monomers and photoinitiators. The second area of concern is the rate of photopolymerization. For SL, the polymerization must occur rapidly to provide enough mechanical robustness to the 3D part as it is built so that it can survive the layer-by-layer writing, and enable its handling when the writing process is complete. This paper reports on early studies related to these issues. In particular, the toxicity of candidate photoinitiators and photoacid generators was studied, and the resulting photopolymerization behavior of photosensitive resins has been investigated.

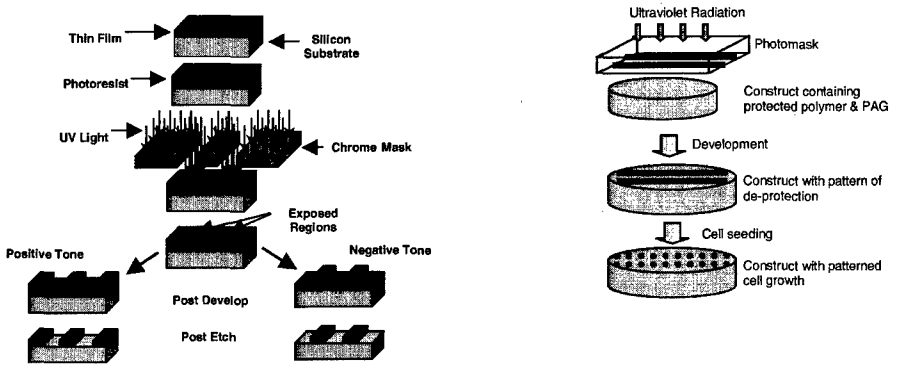

Figure 3. Comparison of standard lithography to surface patterning of a 3D, SL-made construct.

EXPERIMENTAL DETAILS

For the cell compatibility testing, the MC3T3-E1 cell line from American Type Culture Collection (ATCC) was used. The cells were cultured using Alpha minimal essential medium (Cellgro), supplemented with 10% fetal calf serum (ATCC), and 1% penicillin/streptavidin. The cells were grown on standard tissue culture polystyrene plates (Corning). For photoinitiator toxicity tests, the initiators were doped into a biocompatible matrix of poly(caprolactone) (PCL;

Sigma). The radical photoinitiators and photoacid generators used in this study were obtained from Ciba. The PCL pellets and the initiators were all dissolved in chloroform, and the solution was spin cast onto glass cover slips. The cover slips were baked on a hotplate at 110°C for 5 minutes to remove residual casting solvent. Before cell seeding, each sample was sterilized with ethanol, and then rinsed with DPBS containing calcium and magnesium. Cells were seeded onto the glass cover slips in a Corning® Ultra Low Cluster, 6 well polystyrene plate at 7×10^4 cells/ml. The cells were evaluated 24 hours after seeding by staining with Live/Dead Kit L-3224 (Molecular Probes) to visualize and count the cells.

The monomers used for photopolymerization experiments were n-vinyl-2-pyrrolidone (NVP; Polysciences, Inc) and trimethylolpropane triacrylate (Sartomer). The NVP monomer serves as our model compound for the protected NVP monomer that will ultimately be used for dual wavelength patterning. For each sample, 4 wt% photoinitiator (with respect to monomer mass) was used. Photopolymerization experiments were performed on thin films of the monomer solution prepared using spin casting onto glass cover slips. Prior to monomer deposition, the glass surface was primed with a coupling agent, (3-Acryloxypropyl)trimethoxy-silane (Gelest), to aid in film adhesion. Photopolymerization experiments were performed using 365 nm light under a nitrogen atmosphere. Gas chromatography was used to study the residual monomer present in polymerized samples. For these tests, the polymerized samples were extracted in ethanol for 15 minutes, and the resulting ethanol composition was analyzed using a Shimadzu GC 14-A gas chromatograph equipped with an FID detector using an HP-5 column (30 M length, 0.25 mm inner diameter, and 0.25 mm film thickness). The temperature program used was (1) heating from 50 °C to 140 °C at 30 K/min and (2) heating from 140 to 300 °C at 40 K/min under constant pressure with inlet and detector temperatures set constant at 330 °C.

DISCUSSION

One concern in using stereolithography to produce tissue engineering constructs is the toxicity of the chemicals used in the part fabrication. In particular, monomers are known to be toxic, and some photoinitiator molecules have been shown to negatively affect cell growth [9]. During the stereolithography process, it is possible for these small molecules to become trapped in the part as polymerization occurs. A concern then is that these small molecules may slowly leach out of the scaffold and adversely affect cell viability. Thus, the toxicity of the photoinitiators used in this work was quantified. Cells were cultured and evaluated with Live/Dead assay on PCL doped with the various photoinitiators. A live/dead percentage of cells after 24 hours of cell culture was calculated and compared to a control group. The relative survival of cells in the presence of the various photoinitiators is presented in Figure 4. The results from this work show that the Irgacure 651 initiator should be avoided. This agrees with previous studies done on the cytotoxicity of photoinitators [9].

For SL fabrication to be practical, the photopolymerization process must be relatively fast and produce a polymer part with sufficient mechanical integrity to be handled. Therefore, when proposing new SL resin formulations, these issues must be tested. Although the goal is to use the protected NVP monomer previously used in the "bioresist" work by Gonsalves and coworkers, this monomer must be custom synthesized at this point. Therefore, commercially available n-vinyl-2-pyrrolidone (NVP) was used as a model monomer for photopolymerization

studies. Previous experiments have shown that NVP and the protected NVP monomers have similar polymerization characteristics.

Initial photopolymerization experiments using pure NVP-photoinitiator mixtures required extremely large exposure doses on the order of 2000 mJ/cm^2 to produce robust solid films. Since oxygen quenching of radicals was prevented using a nitrogen

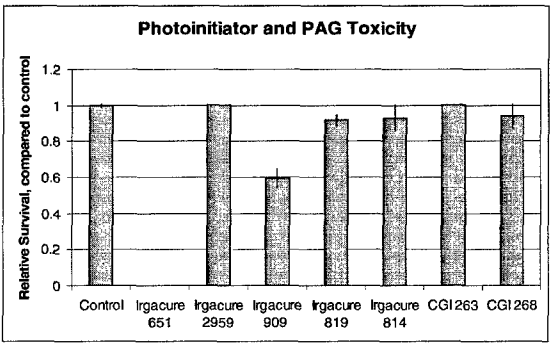

Figure 4. Photoinitiator and PAG toxicity

atmosphere, it was suspected that the major photospeed limitations with the NVP system were the slow polymerization rate of the monomer itself and the fact that it was monofunctional. In order to formulate a resin mixture with a higher sensitivity that could be used in an SL system, co-polymerizations of NVP with an acrylate monomer were investigated. The acrylate monomer chosen is tri-functional, and offers both faster radical polymerization kinetics and the opportunity to produce a highly cross-linked material. The inclusion of the acrylate was shown to reduce the required exposure doses by one to two orders of magnitude. Also, whereas PVP is water-soluble, the acrylate polymer is not. By incorporating the acrylate into the polymer system, we are able to make films that do not immediately degrade upon exposure to water. The affect of varying the NVP:acrylate monomer ratio was also investigated. Samples with high NVP:acrylate ratios (i.e. 80% to 90% NVP) were cloudy, soft, and peeled off the glass very easily. Films with 50% or more acrylate were transparent and more mechanically robust.

For the 30:70 NVP:acrylate formulation, the effects of photopolymerization exposure dose on the amount of residual monomer left in the film were investigated. As a simple test of the amount of leachable residual monomer left in each sample, the samples were extracted in ethanol and GC analysis was performed to quantify the extracted monomer. The resulting data show clearly that longer exposure times yield less leached monomer. Also, for this particular formulation, the data extrapolates to show that a dose of ~40 mJ/cm^2 will yield a polymer that essentially does not leach monomer. Samples made with 100 and 300 mJ/cm^2 doses were also examined, and no detectable monomer was present in the ethanol wash.

Finally, as a preliminary test of biocompatibility, cell growth studies were conducted on photopolymerized NVP-acrylate copolymers. Cells were seeded and grown on a 1:1 NVP:acrylate film. The cells were plated at 3.8 x 10^5 cell/ml and allowed to grow for 5 days. Figure 6 shows an optical micrograph of a region of live cells on a typical sample after 5 days. The growth of these cells was slow as compared to the polystyrene culture dish control, but the absence of significant dead cells demonstrates that the photopolymerized construct is not overly toxic. It was expected that cells would not demonstrate a strong affinity for this model surface since it is the analog for the protected NVP surface. However, it is anticipated that the polymer made with the hydrophilic form of this monomer will demonstrate significantly better cell growth and proliferation. More studies on functionally protected NVP copolymers are in progress.

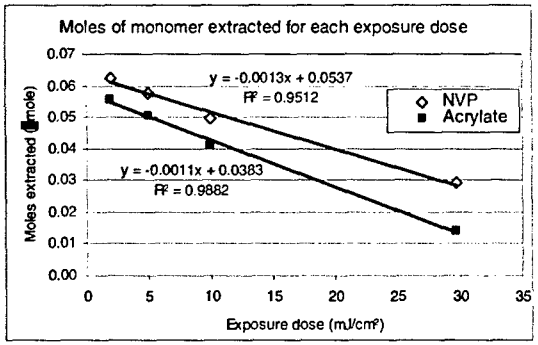

Figure 5: Amount of monomer extracted from photopolymerized NVP-acrylate material as a function of exposure dose.

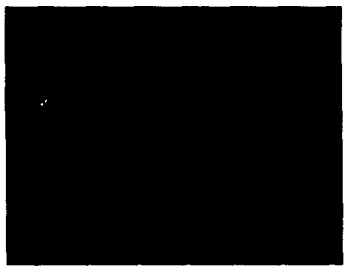

Figure 6: Cells on photopolymerized film visualized with Hoescht nucleus stain

CONCLUSIONS

A new approach for producing 3D hierarchically structured scaffolds via stereolithography, which contain both physical structure and chemical composition control, has been described. Examples of radical photoinitiators and photoacid generators that are biocompatible and can be used with this approach have been identified. Cell growth on photopolymerized surfaces of NVP-acrylate copolymers has been demonstrated. The incorporation of multi-functional acrylate monomers has been shown to improve the photosensitivity of resins containing NVP type monomers. Future studies are focused on investigating the fundamental behavior and processing of photocurable resins containing protected NVP monomers. These materials, in conjunction with dual wavelength SL techniques, will allow for the fabrication of structurally and chemically heterogeneous polymeric scaffolds for tissue engineering.

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